Unveiling the fungal diversity of healthy and infected tea (*Camellia sinensis* L.): Microscopic characterization and internal transcribed spacer-based identification

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

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IN
BIOTECHNOLOGY

Submitted By

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I hereby declare that the work which is being presented in this dissertation entitled "**Unveiling the fungal diversity of healthy and infected tea (*Camellia sinensis* L.): Microscopic characterization and internal transcribed spacer-based identification**" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from January to February under the supervision of Dr. Avishek Banik- Department of Institute of Health Sciences ,Presidency University (2nd Campus)

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LIST OF FIGURES

Serial No.	Title	Page no.
4.2	Microscopic observation of Fungal Isolates	18
4.3.2	Flowchart of Genomic DNA Isolation	19
4.3.3.	Thermocycler	20
5.2(A)	Microscopic observation of IRF	21
5.2(B)	Microscopic observation of HLF	22
5.3	Genomic DNA Concentration	24
5.4	Phylogenetic Tree Constructed using Molecular Evolutionary Genetic Analysis(MEGA v.11) software based on the Neighbour Joining method	25

LIST OF TABLES

Table No.	Title	Page No.
Table 1	Morphological and Microscopic Characteristics of Fungal Isolates from Tea Plants	23-24
Table 2	DNA Quantification and Purity Assessment of Infected Leaf Samples (IRF)	24
Table 3	DNA Quantification and Purity Assessment of Healthy Leaf Samples (HLF)	24

ABBREVIATIONS

ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
IRF	Infected Root Fungi
HLF	Healthy Leaf Fungi
PDB	Potato Dextrose Broth
AMF	Arbuscular Mycorrhizal Fungi
VOC	Volatile Organic Compound
LPCB	Lactophenol Cotton Blue
KOH	Potassium Hydroxide
PDA	Potato Dextrose Agar
µL	Microliter
CFW	Calcofluor White Stain
PGP	Plant Growth Promoting
rRNA	Ribosomal ribonucleic acid

ABSTRACT

Tea (*Camellia sinensis* L.) is a commercially vital crop that frequently suffers from fungal infections, which negatively influence both productivity and quality. This study was undertaken to assess the diversity of fungi colonizing both healthy and diseased leaves and roots of tea plants, employing a combination of morphological techniques and Internal Transcribed Spacer (ITS) region-based molecular identification. Fungal specimens were initially cultured on Potato Dextrose Agar (PDA), and pure isolates were obtained through the cork borer purification method. Microscopic evaluation of fungal features was carried out using lactophenol cotton blue staining in conjunction with the wet mount method, allowing clear visualization of fungal structures such as hyphae, spores, and conidia under a compound light microscope. For molecular identification, selected fungal isolates were grown in Potato Dextrose Broth (PDB) enriched with Tween 80 to facilitate uniform mycelial distribution. Cultures were agitated for 72 hours and then left to incubate under static conditions for four additional days to promote biomass accumulation. Harvested mycelia were processed for genomic DNA extraction using a fungal DNA isolation kit (HiMedia). The ITS region of the ribosomal DNA was amplified using universal primers ITS1 and ITS4. Amplified products were visualized through agarose gel electrophoresis before sequencing. Sequence analysis using the BLAST tool revealed a wide spectrum of fungal taxa.The results affirm the value of integrating classical microscopic methods with ITS-based molecular diagnostics to accurately profile fungal communities in tea plants and highlight the potential implications for crop health and disease management.

KEYWORDS: Tea microbe, Fungi, Internal transcribed spacer (ITS), Microscopy

CONTENTS

1. **Introduction…**.....**Page 10-11**

2. **Review of Literature:**

    2.1. Overview of tea plants and its economic importance….....Page 12

    2.2. Tea associated microbes and their role in tea health….....Page 13

    2.3. Symbiotic and pathogenic fungal interactions in tea plants….....Page 13-14

    2.4. Common fungal diseases affecting tea crops….....Page 14

    2.5. Traditional vs. molecular methods for fungal identification….....Page 14-15

3. **Aims & Objective:**.....**Page 16**

4. **Materials and method:**

    4.1. Purification….....Page 17

    4.2. Microscopy….....Page17

    4.3. Molecular Identification of Fungal Isolates Using ITS Region

        4.3.1. Fungal biomass preparation….....Page 18

        4.3.2. Genomic DNA Extraction….....Page 18-19

        4.3.3. PCR Amplification of ITS region….....Page 19-20

        4.3.4. Construction of Phylogenic Tree….....Page 20

5. **Result and discussions…**.....Page 21-25

6. **Conclusion…**.....Page 26-27

7. **References…**.....Page 28-29

8. **Plagiarism Report…**.....Page 30-38

1. INTRODUCTION

Globally, tea (*Camellia sinensis* L.) is surpassed only by water in terms of overall consumption. Its cultivation is widespread across tropical and subtropical regions and plays a vital role in the agricultural economy of many countries, including India, China, and Sri Lanka along with 50 more countries. India is recognized as the second-largest producer of this crop, with commercially grown plants mainly being hybrids of two main types: Camellia sinensis var. sinensis and Camellia sinensis var. assamica. Tea is well-known for its rich content of health-promoting compounds such as flavonoids, polyphenols, amino acids, alkaloids, and phenolic acids. However, the chemical makeup of tea is influenced by plant genetics, environmental conditions, microbial interactions, and post-harvest processing. Various fungal pathogens, particularly species of the genus Fusarium, are known to infect tea plants, leading to diseases such as dieback, collar canker, leaf spot, and wilt. For instance, Fusarium solani causes stem infections that result in bark splitting, white mould, and eventually death of branches. Fusarium proliferatum causes leaf spot, which appears as brown patches surrounded by yellow borders. Additionally, other fungal pathogens like *Poria hypolateritia* (red root-rot) and *Phomopsis theae* (collar canker) also contribute to tea plant decline by affecting roots and stems, ultimately reducing yield and quality (Tang et al., 2024). Fungi are an essential part of the plant microbiome, colonizing different plant parts in both beneficial and harmful ways. This fungal community, referred to as the "mycobiome," supports plant functions such as nutrient absorption, resistance to environmental stress, immune regulation, and protection from disease. While some fungi form helpful associations, others act as pathogens that can damage crops and reduce productivity.Roots and leaves provide unique habitats for fungal colonization. Root-associated fungi include beneficial mycorrhizae and harmful soil-borne pathogens, which influence nutrient uptake and help plants cope with drought or poor soil. On the other hand, leaf-associated fungi (epiphytes and endophytes) contribute to disease prevention and surface defense mechanisms (Bag et al., 2022).Changes in the structure of these fungal communities can indicate plant health status. Pathogenic fungi often disrupt the balance of beneficial microbes in diseased plants. Comparing the fungal diversity between healthy and infected tissues helps identify harmful or protective species involved in disease processes.Traditional methods like culturing fungi in the lab are limited, as many fungi are unculturable or slow-growing. Molecular techniques have revolutionized fungal identification, especially through DNA-based approaches like

Internal Transcribed Spacer (ITS) sequencing. ITS is a widely used genetic marker that enables precise identification of both known and previously uncharacterized fungi. These tools provide deeper insight into plant-fungal interactions and support better management of crop diseases.

2. REVIEW OF LITERATURE

2.1. Overview of tea plants (*Camellia sinensis* L.) and its economic importance:

The tea plant, scientifically known as *Camellia sinensis* and classified under the Theaceae family, is a perennial evergreen species that can attain a maximum height of approximately 16 meters. It is primarily cultivated for its young leaves, which are processed to produce various types of tea, making it one of the most commercially significant crops worldwide (Aboulwafa et. al., 2019). Tea cultivation depends on various environmental factors, including climate, soil quality, and altitude, with major producers being China, India, Sri Lanka, and Japan (Soni et. al., 2015).

Tea possesses strong antioxidant properties, primarily due to its flavonoid components such as theaflavins and catechins. These antioxidants help combat oxidative stress, which occurs when the balance between free radicals and antioxidants is disrupted, leading to conditions like atherosclerosis, cancer, rheumatoid arthritis, and organ damage. Tea flavonoids act as free radical scavengers, preventing oxidative damage by neutralizing reactive oxygen species and chelating metals like iron and copper. Studies indicate that tea catechins are readily absorbed in the human body, with plasma levels increasing significantly after tea consumption. While some research suggests that adding milk may reduce antioxidant effectiveness due to interactions with milk fat, other studies show no significant impact on catechin bioavailability. Overall, tea consumption contributes to enhanced antioxidant potential and may play a protective role against various diseases.Numerous epidemiological, case-control, and cohort studies have been conducted to examine the relationship between tea consumption and the incidence of cancer in humans, with several researchers reviewing this topic.The beneficial effects of tea are largely attributed to its flavonoid content and antioxidant properties, which help the body counteract oxidizing agents, whether naturally produced or introduced externally. The polyphenols in tea may also reduce the risk of certain cancers by activating phase I and phase II metabolic enzymes, which enhance the breakdown and elimination of carcinogens. Research focused on tea's bioactive components offers a potential strategy for lowering the incidence and mortality rates of various diseases. Additionally, tea remains an affordable, naturally sourced beverage, making it a healthier alternative to modern soft drinks (Sharma et. Al., 2007).

2.2. Tea associated microbes and their role in tea health:

The growth and quality of tea are greatly affected by the microbial communities linked to tea plants (Bag et. al., 2022). The rhizosphere, phyllosphere, and root zones are primarily inhabited by microbial phyla such as Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes (Tang et. al., 2024). Microorganisms play a vital role in enhancing tea quality by transforming natural compounds during fermentation. Certain fungal species, like *Eurotium cristatum*, contribute to flavor, color, and aroma development by modifying phenolic compounds. Beneficial bacteria, such as *Bacillus coagulans*, are known for their probiotic properties and are used to improve both the taste and health benefits of fermented teas like pu-erh. These microbes produce enzymes and bioactive substances that influence tea's functional and sensory attributes. Advanced microbiological and sequencing methods are essential to isolate and identify these beneficial strains. Fermented teas such as kombucha and fuzhuan brick tea are gaining popularity due to their antioxidant, antimicrobial, and metabolic health benefits. Despite growing interest, comprehensive studies focusing on the microbial dynamics and their functional roles in various fermented teas remain limited (Assad et. al., 2023).

2.3. Symbiotic and pathogenic fungal interactions in tea plants:

Symbiotic and pathogenic fungal interactions in tea plants (*Camellia sinensis* L.) significantly influence their growth, yield, and overall health. These fungi facilitate nutrient absorption and contribute to plant growth promotion. Studies show that AMF improves leaf quality by increasing amino acids, sugars, proteins, polyphenols, and flavonoids, likely through upregulation of related metabolic genes (Bag et. al., 2022). The rhizosphere, endosphere, and phyllosphere of tea plants support a complex and diverse microbial ecosystem that includes both symbiotic and pathogenic fungi. These fungi play crucial roles in nutrient exchange, growth promotion, and disease development. Dominant fungal groups such as *Fusarium*, *Trichoderma*, *Penicillium*, and *Aspergillus* can either enhance plant health through mutualistic associations or contribute to diseases. Endophytic and rhizospheric fungi, including arbuscular mycorrhizal fungi (AMF), not only improve nutrient uptake but also suppress pathogens. The composition of these communities is influenced by plant tissue type,

soil pH, and environmental conditions, shaping the overall health and productivity of tea plants (Mondal et. al., 2025).

2.4. Common fungal diseases affecting tea crops:

Some of the fungal diseases are Blister blight, Gray blight, Brown blight, Twig dieback, Stem cankers, Root rot etc. (Tang et. al., 2024).Blister blight disease that mainly affects the young and tender leaves of tea plants, The fungus responsible for this disease has a short life cycle (Sen et. al., 2020). Gray blight is a prevalent disease in tea plants, triggered by the fungal pathogen *Pestalotiopsis theae*, and is found in many tea-growing regions. It often starts through wounds from tools and affects older leaves that support new growth, leading to major yield loss. The disease shows as brown spots that turn grey with brown edges and gradually spread. Detecting it in large, hilly plantations is challenging, and traditional methods are time-consuming and often inaccurate, leading to wrong fungicide use and increased costs (Pandian et. al., 2023). Brown blight is caused by *Colletotrichum* spp. It begins as dark-green, water-soaked spots on leaves, which grow into reddish-brown patches with black acervuli. These structures produce conidia, helping the disease spread (Lin et. al., 2023). Tea dieback disease, caused by *Fusarium solani*, by which most leaves gradually become death. Symptoms include small or yellowing leaves, poor twig growth, early leaf drop, and leaf edge burn, eventually causing plant decline (Devi et. al., 2012). Tea plants are susceptible to several types of canker diseases, most of which are caused by fungal pathogens. Fungal species like *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, and *Diaporthe longicolla* have been identified as canker-causing agents in tea and other crops in various parts of the world, although there are no documented cases of these fungi affecting tea plants in India (Pandey et. al., 2024).

2.5. Traditional vs. molecular methods for fungal identification:

Fungal identification is crucial for understanding the diversity associated with tea (Camellia sinensis L.). Classical methods, based on morphological and cultural characteristics, have long been used but often lack precision for species-level identification. Modern molecular techniques, especially ITS region sequencing, offer greater accuracy and can uncover cryptic species. This study integrates both approaches, microscopic and molecular based, and provides a comprehensive understanding of fungi in healthy and infected tea tissues. Despite advancements, molecular data remain limited, highlighting the need for more extensive

studies in this area (Gautam et. al., 2022). Microscopic observation of fungi is an essential method for identifying and studying their structural characteristics. The microscopic appearance of fungi is observed using various staining techniques such as Potassium Hydroxide (KOH) mount, Lactophenol Cotton Blue stain (LPCB), Gram stain, and .The slide culture technique is particularly useful for preserving the natural arrangement of fungal reproductive structures, making it easier to study their morphology without significant disruption. Commonly observed fungi under the microscope include *Aspergillus, Penicillium, Rhizopus, Mucor, Fusarium, Trichophyton, Microsporum,* and *Epidermophyton*. But there are certain limitations of traditional technique also that is morphological variability, expert knowledge required, time consuming. Molecular identification is preferred over traditional techniques for fungal identification because it offers significantly higher accuracy, speed, and sensitivity, allowing for precise species identification even when morphological features are ambiguous or when dealing with small sample quantities, which is often the case with cryptic or poorly characterized fungal species; whereas traditional methods rely heavily on visual observation of morphological characteristics which can be variable and prone to misinterpretation. DNA barcoding has evolved into a standardised method for evaluating patterns of biodiversity worldwide and may diagnose both known and undiscovered species for non-taxonomists. Short DNA sequences are used in DNA barcoding, a quick, precise, and standardised technique for species-level identification. The internal transcribed spacer (ITS) is a non-coding RNA region situated between the structural ribosomal RNA (rRNA) genes within a single precursor transcript, running in the 5' to 3' direction. It comprises the 18S small subunit, the 5.8S rRNA, and the 28S large subunit, with the ITS1 and ITS2 regions separating these rRNA genes. Due to its high copy number, the ITS region is easily amplified even from minimal DNA samples, making it a widely used marker in taxonomy and molecular phylogenetics. It exhibits significant variation even among closely related species, making it a valuable tool for species identification.. It exists in multiple copies within most fungal cells and can be consistently amplified using standardized primers. Because of its sensitivity and the widespread use of these primers, a comprehensive reference database is available in GenBank.

3. AIMS & OBJECTIVES

1. Purification of fungal isolates obtained from healthy leaves and infected roots.
2. Characterization of morphological features using light microscopy.
3. Molecular based identification and phylogeny analysis of fungal isolates using ITS rDNA sequencing,

4. MATERIALS AND METHODS

4.1. Purification:

Fungal purification was carried out using the cork borer method to isolate a single fungal species from mixed growth. After initial isolation on Potato Dextrose Agar (PDA) plates, the plates were incubated at 25 ± 2°C for 5-7 days to allow for the development of fungal colonies. Once growth was established, distinct fungal colonies were visually identified based on their morphological characteristics such as color, texture, and growth pattern. A sterile cork borer was used to aseptically cut and transfer a circular disc from the actively growing margin of the selected fungal colony to a fresh PDA plate. This process helps in isolating the actively growing and uncontaminated portion of the fungus. The sub-cultured plates were incubated again under the same conditions until a uniform fungal growth was observed. The procedure was repeated many times to ensure complete purification and removal of any possible contaminants. The isolated fungal cultures were preserved at 4°C for subsequent identification and experimental applications.

4.2. Microscopy:

Microscopy is crucial in fungal identification as it allows for the detailed observation of morphological characteristics such as hyphal structure, spore formation, and reproductive bodies, which are key for distinguishing fungal species. Techniques like Lactophenol Cotton Blue, Calcofluor White, and India Ink staining enhance visualization of fungal cell walls, spores, and capsules, aiding in differentiation from other microorganisms. The microscopic examination of the purified fungal isolates was performed using the Scotch tape preparation method with Lactophenol Cotton Blue (LPCB) staining. A clean glass slide was prepared by placing a single drop of LPCB stain in the center. A small piece of sterile transparent scotch tape was gently pressed onto the surface of a 7 day-old fungal culture to lift hyphae and spores.The adhesive tape bearing fungal structures was gently pressed, adhesive side down, onto a drop of Lactophenol Cotton Blue (LPCB) on a microscope slide to create a wet mount. The slide was then examined under a compound light microscope at 100x magnification.. The morphological features such as hyphal structure, conidiophores, and conidia were recorded to assist in the preliminary identification of the fungal isolates based on microscopic characteristics.

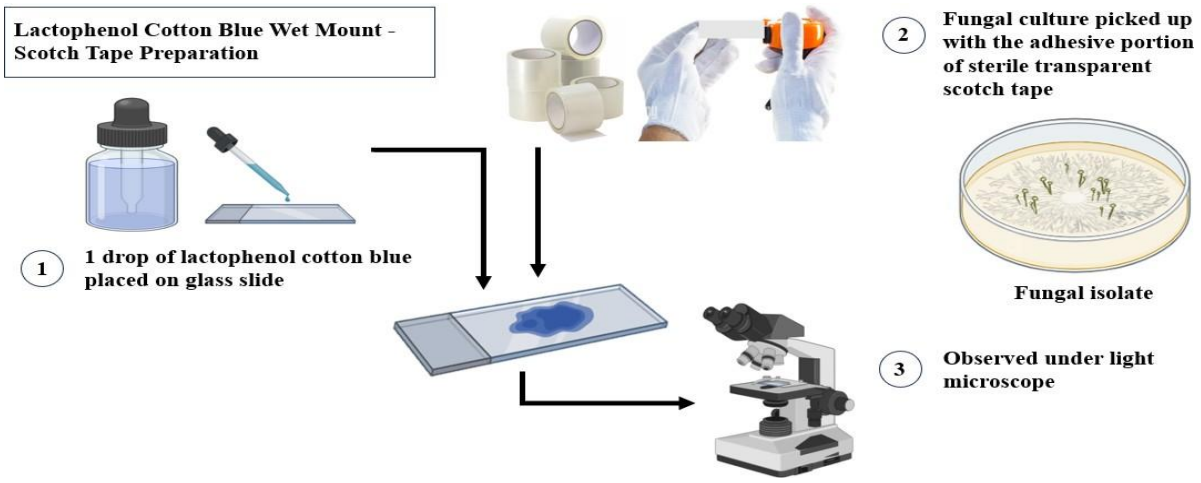

Lactophenol Cotton Blue Wet Mount - Scotch Tape Preparation
1
1 drop of lactophenol cotton blue placed on glass slide
2
Fungal culture picked up with the adhesive portion of sterile transparent scotch tape
Fungal isolate
3
Observed under light microscope

Fig. 4.2. Microscopic observation of Fungal Isolates

4.3. Molecular Identification of Fungal Isolates Using ITS Region

Molecular identification of purified fungal isolates was performed through amplification and sequencing of the Internal Transcribed Spacer (ITS) region of fungal rDNA, a highly conserved region used for fungal taxonomy and identification.

4.3.1 Fungal biomass preparation

A 5 mm disc from a purified fungal colony was cut using a sterile cork borer and inoculated into 100 mL of sterile Potato Dextrose Broth (PDB) in a 250 mL conical flask with Tween 80 to prevent clumping and promote uniform dispersion of fungal hyphae in the liquid medium. The culture was incubated at 25 ± 2°C with shaking at 120 rpm for 3 days, followed by static incubation for 4 days. After 7 days, the mycelial mat was crushed manually using a sterile mortar and pestle for DNA extraction.

4.3.2 Genomic DNA extraction

Genomic DNA was extracted utilizing the HiMedia® Fungal Genomic DNA Miniprep Purification Kit, following the protocol provided by the manufacturer. Initially, the fungal mycelium was grinded thoroughly using a rough mortar and pestle. Subsequently, a lysis buffer was applied to break down the cells. This step was complemented by enzymatic treatment to further facilitate cell disruption.DNA purification was carried out using a column-based protocol.. Finally, the purified DNA was eluted using an elution buffer and

stored at -20°C for future use.The concentration and purity of the extracted DNA were assessed using spectrophotometric absorbance measurements.

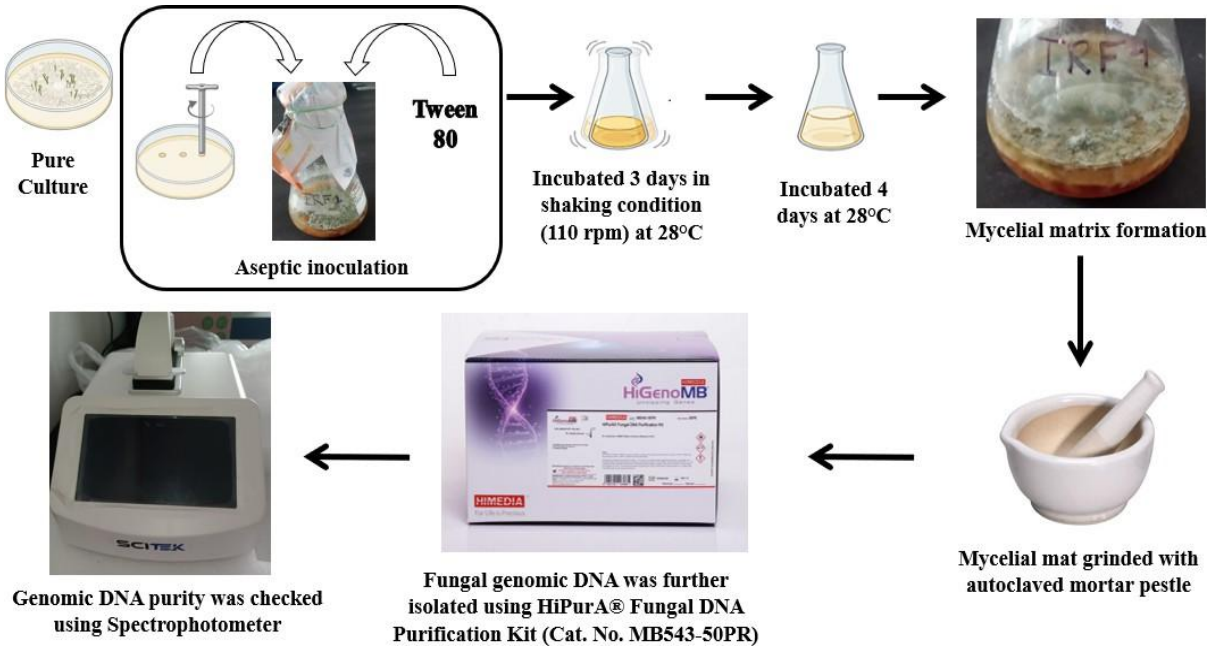

Pure Culture
Tween 80
Aseptic inoculation
Incubated 3 days in shaking condition (110 rpm) at 28°C
Incubated 4 days at 28°C
Mycelial matrix formation
Mycelial mat grinded with autoclaved mortar pestle
HiGenoMB
HIMEDIA
Fungal genomic DNA was further isolated using HiPurA® Fungal DNA Purification Kit (Cat. No. MB543-50PR)
SCITEK
Genomic DNA purity was checked using Spectrophotometer

Fig . 4.3.2. Flowchart of Genomic DNA Isolation

4.3.3 PCR Amplification of ITS region

The **ITS region** of the rDNA was amplified using the **ITS1** and **ITS4** primers:

- **ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')**
- **ITS4 (5'-TCCTCCGCTTATTGATATGC-3')**

The PCR mixture was made by mixing the DNA template, forward and reverse primers, PCR master mix, and sterile distilled water in proper amounts.

- 25µL Hi-PCR® REDy Master Mix
- 1µL forward primer (ITS1).
- 1µL reverse primer (ITS4).
- 2.5µL of genomic DNA template.
- Remaining volume made up with molecular-grade water to a final volume of 50 µL.

The PCR was conducted using the following thermal cycling conditions:

- Initial denaturation: 94°C for 5 minutes.
- 35 cycles of:
    - Denaturation: 94°C for 30 seconds
    - Annealing: 55°C for 30 seconds
    - Extension: 72°C for 1 minute.
- Final extension: 72°C for 10 minutes

In 1.5%agarose gel with ethidium bromide, the samples were run to check the PCR result, in UV light, DNA bands had been checked.

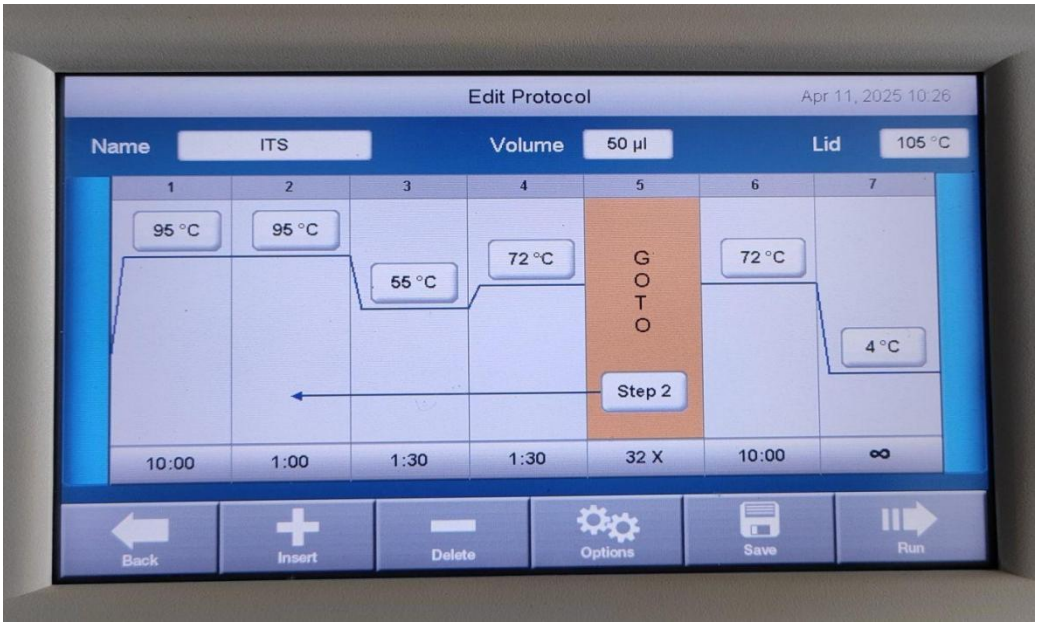


Fig. 4.3.3 Thermocycler

4.4. Construction of Phylogenetic tree:

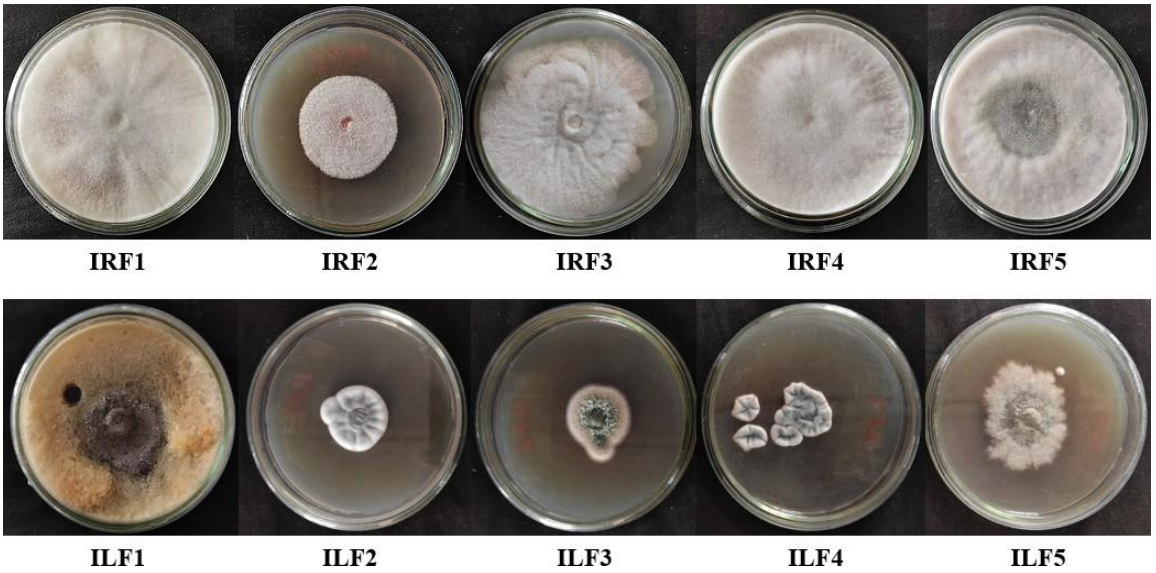
Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA v.11) software based on the Neighbour Joining method.

5. RESULT AND DISCUSSION

1. Purification:

The purification process resulted in clear and distinct fungal colonies, as shown in the images. This confirms the effectiveness of the method in isolating pure fungal cultures from the mixed samples. Purified fungi are crucial for accurate identification and further study.

Fig 5.1:Purified Fungal Isolates



2. Microscopy:

Microscopic examination of the purified fungal isolates revealed characteristic structures such as hyphae, spores, and conidia. These observations helped confirm the morphological identity of the fungi and supported the purification results.

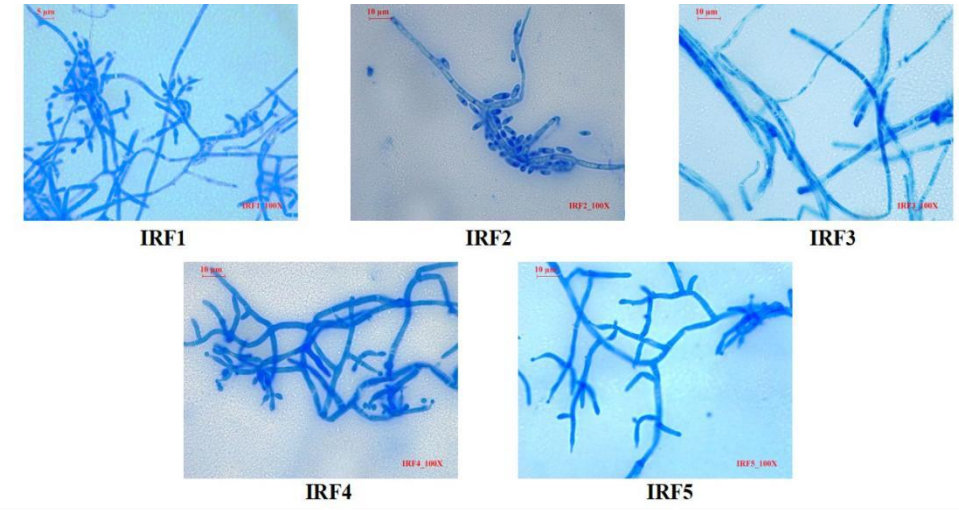


Fig 5.2(A): Microscopic Observation of IRF

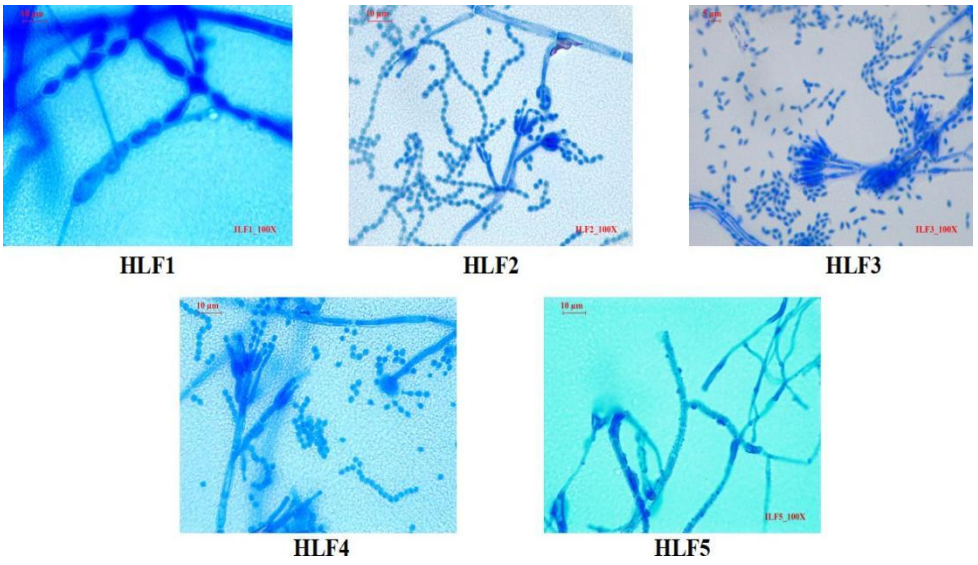


Fig 5.2(B): Microscopic Observation of HLF

Table 1 : Morphological and Microscopic Characteristics of Fungal Isolates from Tea Plants

Fungal isolates	Colonial characteristics	Type of hyphae	Type of spore	Appearance of spore head	Probable organism (based on microscopic observation)
IRF1	Rapid growth; white to green colonies; woolly or compact	Septate, hyaline	Conidia; rare ascospores	Brush-like clusters of conidia	**Trichoderma sp.**
IRF2	Cottony; white to pink or purple centers; fast growing	Septate, hyaline	Macroconidia , microconidia,	Canoe-shaped macroconidia	**Fusarium sp.**
IRF3	White to pinkish colonies; turns reddish; slow to moderate growth	Septate, hyaline	Conidia; ascospores in perithecia	simple or branched heads	**Nectria sp.**
IRF4	Rapid growth; white to green colonies; woolly	Septate, hyaline	Conidia; rare ascospores	clusters of conidia	**Trichoderma sp.**
IRF5	Rapid growth; white to green colonies;	Septate, hyaline	Conidia; rare ascospores	Brush-like clusters of	**Trichoderma sp.**

	woolly or compact			conidia	
HLF1	Colonies pale to tan; slow growing; may show pigmented structures in older cultures	Septate, hyaline	Ascospores in cleistothecia	Globuse fruiting body	**Gelasinospora sp.**
HLF2	Velvety to powdery; blue-green with white edges;	Septate, hyaline	Conidia	Brush-like ; conidia in chains	**Penicillium sp.**
HLF3	Greenish or reddish colonies; powdery; grows moderately fast	Septate, hyaline	Conidia, ascospores	Brush-like with terminal conidia	**Talaromyces sp.**
HLF4	Velvety to powdery; blue-green with white edges; moderate to rapid growth	Septate, hyaline	Conidia	Brush-like (penicillus); conidia in chains	**Penicillium sp.**
HLF5	Woolly to powdery; greyish or olive colonies; slow growing	Septate, hyaline	Conidia	Irregularly branched with nodular swellings	**Nodulisporium sp.**

3. Genomic DNA Concentration:

Table 2: DNA Quantification and Purity Assessment of Infected Leaf Samples (IRF)

Sample ID	Initial DNA Conc. (ng/µL)	Post Gel Conc. (ng/µL)	A260/A280 Ratio	A260/230 Ratio
IRF 1	1100.00	777.7	~1.8	~2.0
IRF 2	499.1	468.9	~1.8	~2.1
IRF 3	13.2	36.8	~1.6	~2.0
IRF 4	886.6	501.8	~1.9	~2.2
IRF 5	280.95	429.8	~1.7	~1.9

Table 3:DNA Quantification and Purity Assessment of Healthy Leaf Samples (HLF)

Sample ID	Initial DNA Conc. (ng/µL)	Post Gel Conc. (ng/µL)	A260/A280 Ratio	A260/230 Ratio
HLF 1	298.0	828.5	~1.8	~2.0
HLF 2	269.5	807.1	~1.7	~2.0
HLF 3	667.1	892.7	~1.8	~2.1
HLF 4	75.5	745.2	~1.7	~2.1
HLF 5	195.8	689.6	~1.6	~2.2

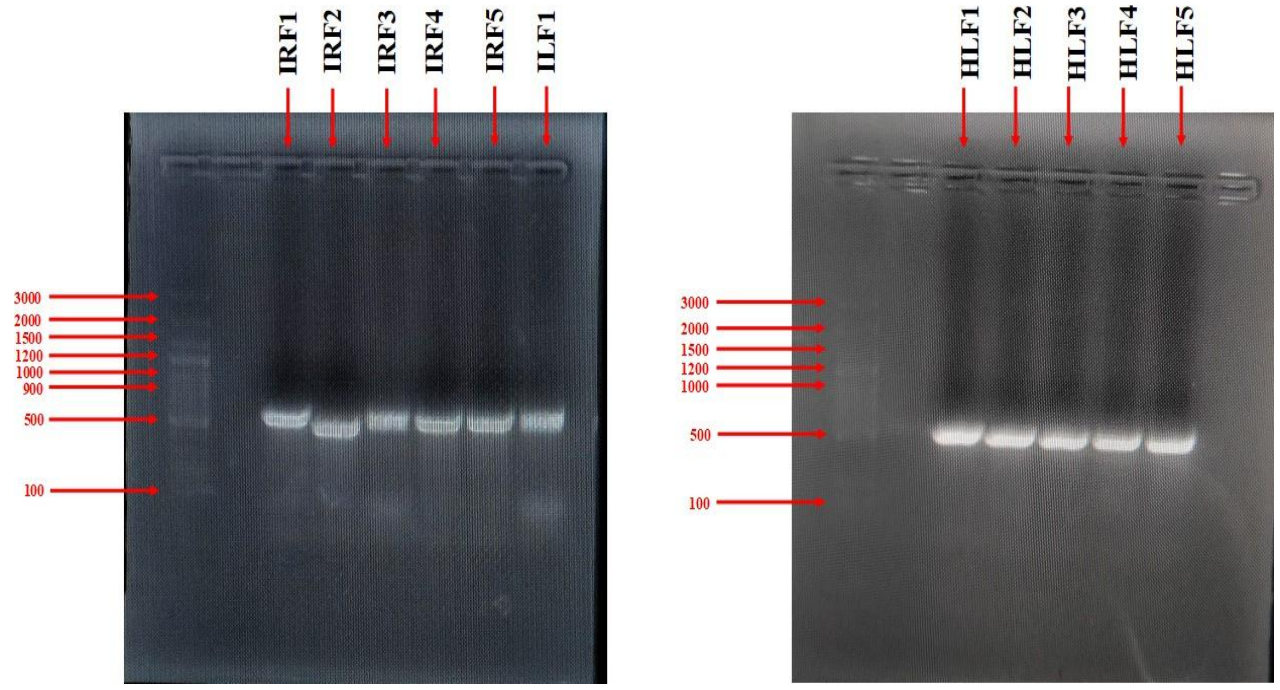


Fig 5.3: Genomic DNA Concentration

The above tables present DNA concentration data for infected (IRF) and healthy (HLF) tea leaf samples before and after gel purification. A notable increase in post-gel concentrations is observed in HLF samples, suggesting efficient DNA recovery. The A260/A280 ratios (~1.6–1.9) and A260/230 ratios (~1.9–2.2) across both sets indicate good DNA purity, suitable for molecular applications like PCR and sequencing.

4. Sequencing & Phylogeny analysis:

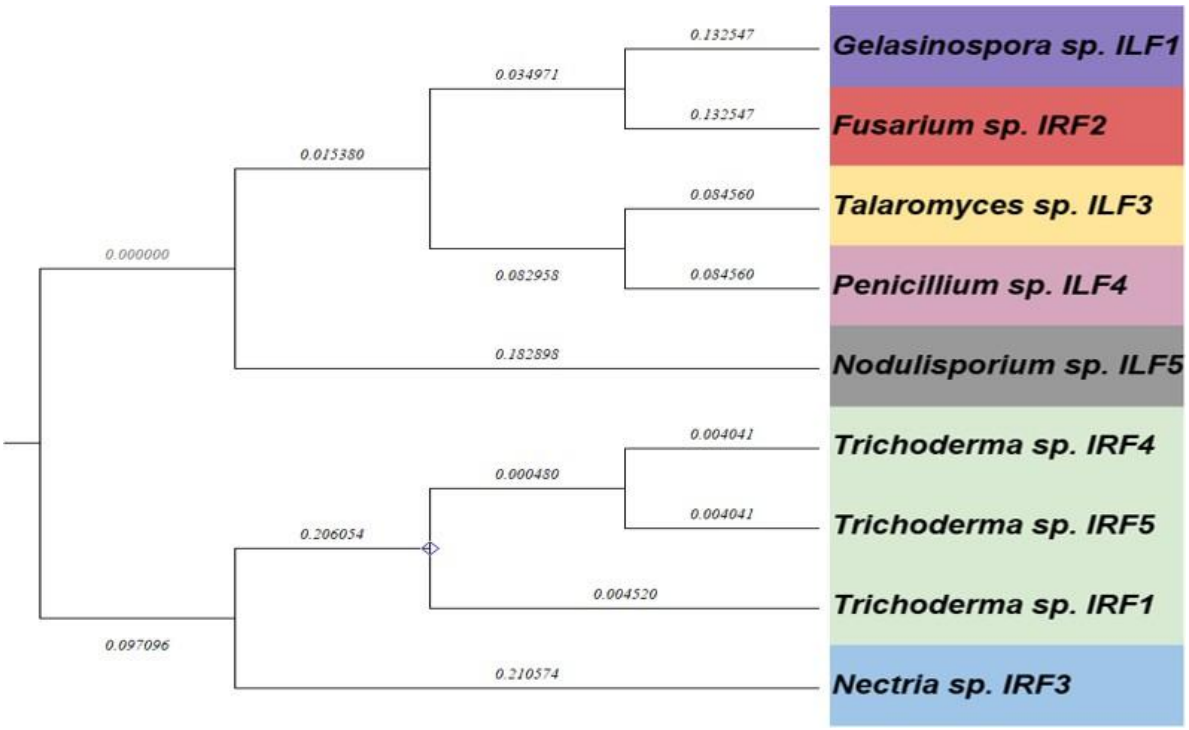


Fig 5.4: Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA v.11) software based on the Neighbour Joining method.

The evolutionary relationships among the fungal isolates were analyzed using ITS sequence data. The tree reveals distinct clustering of genera such as *Trichoderma*, *Penicillium*, *Talaromyces*, *Fusarium*, and *Nectria*, indicating genetic divergence among the isolates from healthy and infected tea samples. Branch lengths represent genetic distances, and closely related species are grouped together, reflecting their phylogenetic proximity.

CONCLUSION

This study effectively revealed the complex fungal diversity associated with healthy and infected tea plants (*Camellia sinensis* L.), focusing on both leaves and roots, by employing a dual approach involving morphological and molecular techniques. Morphological examination was initially carried out using lactophenol cotton blue staining and the cellotape mount method, which provided foundational insights into the structural characteristics of the isolated fungi. However, since morphological identification can be limited by similarities in physical features among different fungal taxa, molecular characterization using Internal Transcribed Spacer (ITS) region amplification was adopted for accurate species-level identification.

The ITS-based sequencing and phylogenetic analysis unveiled a broad spectrum of fungal genera, reflecting the ecological diversity and functional roles of fungi in tea ecosystems. The identified fungi included ***Gelasinospora sp., Fusarium sp., Talaromyces sp., Penicillium sp., Nodulisporium sp., Trichoderma sp.,*** and ***Nectria sp.*** Among these, several species are recognized pathogens, such as *Fusarium solani* and *Fusarium proliferatum*, which are known to cause dieback, collar canker, and leaf spot diseases. Additionally, *Phomopsis theae* and *Poria hypolateritia* were associated with collar stem canker and red root rot, respectively. These diseases are significant threats to tea plantations, leading to reduced plant vigor, lower yield, and compromised leaf quality, which in turn affect both domestic markets and export potential.

Interestingly, the presence of **multiple Trichoderma species**, known for their antagonistic and biocontrol properties, highlights the coexistence of both pathogenic and beneficial fungi within the tea plant microbiome. These beneficial fungi may play a role in natural disease suppression, enhancing plant resilience and promoting healthier growth. Their identification provides potential for further exploration into their application as biological control agents against destructive fungal pathogens.

This research emphasizes the importance of integrating traditional fungal observation techniques with advanced molecular tools to achieve comprehensive mycological profiling. Traditional methods remain valuable for preliminary screening, but molecular analysis allows

researchers to overcome the limitations of culture-based identification, especially for non-sporulating, cryptic, or unculturable fungi.

Understanding the tea plant's mycobiome—the community of associated fungi—can lead to the development of more targeted and eco-friendly disease management strategies. By identifying not only harmful fungi but also beneficial and neutral endophytes, this study sets the stage for future research into plant-fungal interactions, microbial ecology, and their implications in sustainable agriculture.

Moreover, the insights gained from this study can assist in developing early detection systems for fungal pathogens, improving integrated pest management (IPM) protocols, and guiding breeders in selecting disease-resistant tea cultivars. These advances are especially crucial as climate change and intensive farming practices increase the vulnerability of crops to disease outbreaks.

In conclusion, this investigation provides a significant contribution to the understanding of fungal biodiversity in tea plants. It demonstrates how modern molecular biology tools, when combined with classical taxonomy, can unveil hidden microbial dynamics, ultimately aiding in enhancing tea crop health, productivity, and sustainability.

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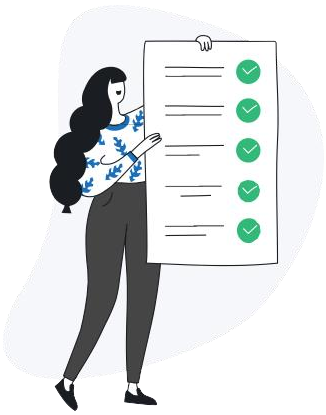
Globally, tea (Camellia sinensis L.) is surpassed only by water in terms of overall consumption. Its cultivation is widespread across tropical and subtropical regions and plays a vital role in the agricultural economy of many countries, including India, China, and Sri Lanka along with 50 more countries. India is recognized as the second-largest producer of this crop, with commercially grown plants mainly being hybrids of two main types: Camellia sinensis var. sinensis and Camellia sinensis var. assamica. Tea is well-known for its rich content of health-promoting compounds such as avonoids, polyphenols, amino acids, alkaloids, and phenolic acids. However, the chemical makeup of tea is in uenced by plant genetics, environmental conditions, microbial interactions, and post-harvest processing. Various fungal pathogens, particularly species of the genus Fusarium, are known to infect tea plants, leading to diseases such as dieback, collar canker, leaf spot, and wilt. For instance, Fusarium solani causes stem infections that result in bark splitting, white mould, and eventually death of branches. Fusarium proliferatum causes leaf spot, which appears as brown patches surrounded by yellow borders. Additionally, other fungal pathogens like Poria hypolateritia (red root-rot) and Phomopsis theae (collar canker) also contribute to tea plant decline by affecting roots and stems, ultimately reducing yield and quality (Tang et al., 2024). Fungi are an essential part of the plant microbiome, colonizing different plant parts in both bene cial and harmful ways. This fungal community, referred to as the “mycobiome,” supports plant functions such as nutrient absorption, resistance to environmental stress, immune regulation, and protection from disease. While some fungi form helpful associations, others act as pathogens that can damage crops and reduce productivity.Roots and leaves provide unique habitats for fungal colonization. Root-associated fungi include bene cial mycorrhizae and harmful soil-borne pathogens, which in uence nutrient uptake and help plants cope with drought or poor soil. On the other hand, leaf-associated fungi (epiphytes and endophytes) contribute to disease prevention and surface defense mechanisms (Bag et al., 2022).Changes in the structure of these fungal communities can indicate plant health status. Pathogenic fungi often disrupt the balance of bene cial microbes in diseased plants. Comparing the fungal diversity between healthy and infected tissues helps identify harmful or protective species involved in disease processes.Traditional methods like culturing fungi in the lab are limited, as many fungi are unculturable or slow-growing. Molecular techniques have revolutionized fungal identi cation, especially through DNA-based approaches like Internal Transcribed Spacer (ITS) sequencing. ITS is a widely used genetic marker that enables precise identi cation of both known and previously uncharacterized fungi. These tools provide deeper insight into plant-fungal interactions and support better management of crop diseases.

REVIEW OF LITERATURE

2.1. Overview of tea plants (Camellia sinensis L.) and its economic importance:

The tea plant, scienti cally known as Camellia sinensis and classi ed under the Theaceae family, is a perennial evergreen species that can attain a maximum height of approximately 16 meters. It is primarily cultivated for its young leaves, which are processed to produce various types of tea, making it one of the most commercially signi cant crops worldwide (Aboulwafa et. al., 2019). Tea cultivation depends on various environmental factors, including climate, soil quality, and altitude, with major producers being China, India, Sri Lanka, and Japan (Soni et. al., 2015).

Tea possesses strong antioxidant properties, primarily due to its avonoid components such as thea avins and catechins. These antioxidants help combat oxidative stress, which occurs when the balance between free radicals and antioxidants is disrupted, leading to conditions like atherosclerosis, cancer, rheumatoid arthritis, and organ damage. Tea avonoids act as free radical scavengers, preventing oxidative damage by neutralizing reactive oxygen species and chelating metals like iron and copper. Studies indicate that tea catechins are readily absorbed in the human body, with plasma levels increasing signi cantly after tea consumption. While some research suggests that adding milk may reduce antioxidant effectiveness due to interactions with milk fat, other studies show no signi cant impact on catechin bioavailability. Overall, tea consumption contributes to enhanced antioxidant potential and may play a protective role against various diseases.Numerous epidemiological, case-control, and cohort studies have been conducted to examine the relationship between tea consumption and the incidence of cancer in humans, with several researchers reviewing this topic.The bene cial effects of tea are largely attributed to its avonoid content and antioxidant properties, which help the body counteract oxidizing agents, whether naturally produced or introduced externally. The polyphenols in tea may also reduce the risk of certain cancers by activating phase I and phase II metabolic enzymes, which enhance the breakdown and elimination of carcinogens. Research focused on tea's bioactive components offers a potential strategy for lowering the incidence and mortality rates of various diseases. Additionally, tea remains an affordable, naturally sourced beverage, making it a healthier alternative to modern soft drinks (Sharma et. Al., 2007).



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2.2.Tea associated microbes and their role in tea health:

The growth and quality of tea are greatly affected by the microbial communities linked to tea plants (Bag et. al., 2022). The rhizosphere, phyllosphere, and root zones are primarily inhabited by microbial phyla such as Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes (Tang et. al., 2024). Microorganisms play a vital role in enhancing tea quality by transforming natural compounds during fermentation. Certain fungal species, like Eurotium cristatum, contribute to avor, color, and aroma development by modifying phenolic compounds. Bene cial bacteria, such as Bacillus coagulans, are known for their probiotic properties and are used to improve both the taste and health bene ts of fermented teas like pu-erh. These microbes produce enzymes and bioactive substances that in uence tea's functional and sensory attributes. Advanced microbiological and sequencing methods are essential to isolate and identify these bene cial strains. Fermented teas such as kombucha and fuzhuan brick tea are gaining popularity due to their antioxidant, antimicrobial, and metabolic health bene ts. Despite growing interest, comprehensive studies focusing on the microbial dynamics and their functional roles in various fermented teas remain limited (Assad et. al., 2023).

Symbiotic and pathogenic fungal interactions in tea plants (Camellia sinensis L.) signi cantly in uence their growth, yield, and overall health. Studies show that AMF improves leaf quality by increasing amino acids, sugars, proteins, polyphenols, and avonoids, likely through upregulation of related metabolic genes (Bag et. al., 2022). The rhizosphere, endosphere, and phyllosphere of tea plants support a complex and diverse microbial ecosystem that includes both symbiotic and pathogenic fungi. These fungi play crucial roles in nutrient exchange, growth promotion, and disease development. Dominant fungal groups such as Fusarium, Trichoderma, Penicillium, and Aspergillus can either enhance plant health through mutualistic associations or contribute to diseases. Endophytic and rhizospheric fungi, including arbuscular mycorrhizal fungi (AMF), not only improve nutrient uptake but also suppress pathogens. The composition of these communities is in uenced by plant tissue type, soil pH, and environmental conditions, shaping the overall health and productivity of tea plants (Mondal et. al., 2025).

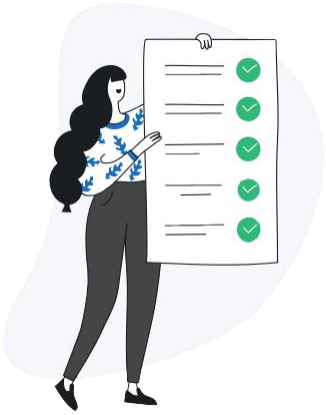
2.4. Common fungal diseases affecting tea crops:

Some of the fungal diseases are Blister blight, Gray blight, Brown blight, Twig dieback, Stem cankers, Root rot etc. (Tang et. al., 2024).Blister blight disease that mainly affects the young and tender leaves of tea plants, The fungus responsible for this disease has a short life cycle (Sen et. al., 2020). Gray blight is a prevalent disease in tea plants, triggered by the fungal pathogen Pestalotiopsis theae, and is found in many tea-growing regions. It often starts through wounds from tools and affects older

leaves that support new growth, leading to major yield loss. The disease shows as brown spots that turn grey with brown edges and gradually spread. Detecting it in large, hilly plantations is challenging, and traditional methods are time-consuming and often inaccurate, leading to wrong fungicide use and increased costs (Pandian et. al., 2023). Brown blight is caused by Colletotrichum spp. It begins as dark-green, water-soaked spots on leaves, which grow into reddish-brown patches with black acervuli. These structures produce conidia, helping the disease spread (Lin et. al., 2023). Tea dieback disease, caused by Fusarium solani, by which most leaves gradually become death. Symptoms include small or yellowing leaves, poor twig growth, early leaf drop, and leaf edge burn, eventually causing plant decline (Devi et. al., 2012). Tea plants are susceptible to several types of canker diseases, most of which are caused by fungal pathogens. Fungal species like Botryosphaeria dothidea, Lasiodiplodia theobromae, and Diaporthe longicolla have been identi ed as canker-causing agents in tea and other crops in various parts of the world, although there are no documented cases of these fungi affecting tea plants in India (Pandey et. al., 2024).

2.5. Traditional vs. molecular methods for fungal identi cation:

Fungal identi cation is crucial for understanding the diversity associated with tea (Camellia sinensis L.). Classical methods, based on morphological and cultural characteristics, have long been used but often lack precision for species-level identi cation. Modern molecular techniques, especially ITS region sequencing, offer greater accuracy and can uncover cryptic species. This study integrates both approaches, microscopic and molecular based, and provides a comprehensive understanding of fungi in healthy and infected tea tissues. Despite advancements, molecular data remain limited, highlighting the need for more extensive studies in this area (Gautam et. al., 2022). Microscopic observation of fungi is an essential method for identifying and studying their structural characteristics. The microscopic appearance of fungi is observed using various staining techniques such as Potassium Hydroxide (KOH) mount, Lactophenol Cotton Blue stain (LPCB), Gram stain, and .The slide culture technique is particularly useful for preserving the natural arrangement of fungal reproductive structures, making it easier to study their morphology without signi cant disruption. Commonly observed fungi under the microscope include Aspergillus, Penicillium, Rhizopus, Mucor, Fusarium, Trichophyton, Microsporum, and Epidermophyton. But there are certain limitations of traditional technique also that is morphological variability, expert knowledge required, time consuming. Molecular identi cation is preferred over traditional techniques for fungal identi cation because it offers signi cantly higher accuracy, speed, and sensitivity, allowing for precise species identi cation even when morphological features are ambiguous or when dealing with small sample quantities, which is often the case with cryptic or poorly characterized fungal species; whereas traditional methods rely heavily on visual observation of morphological characteristics which can be variable and prone to misinterpretation. DNA barcoding has evolved into a standardised method for evaluating patterns of biodiversity worldwide and may diagnose both known and undiscovered species for non-taxonomists. Short DNA sequences are used in DNA barcoding, a quick, precise, and standardised technique for species-level identi cation.



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The internal transcribed spacer (ITS) is a non-coding RNA region situated between the structural ribosomal RNA (rRNA) genes within a single precursor transcript, running in the 5' to 3' direction. It comprises the 18S small subunit, the 5.8S rRNA, and the 28S large subunit, Due to its high copy number, the ITS region is easily ampli ed even from minimal DNA samples, making it a widely used marker in taxonomy and molecular phylogenetics. It exhibits signi cant variation even among closely related species, making it a valuable tool for species identi cation.. It exists in multiple copies within most fungal cells and can be consistently ampli ed using standardized primers. Because of its sensitivity and the widespread use of these primers, a comprehensive reference database is available in GenBank.

AIMS & OBJECTIVES

Puri cation of fungal isolates obtained from healthy leaves and infected roots.

Characterization of morphological features using light microscopy

4.1. Puri cation:

Fungal puri cation was carried out using the cork borer method to isolate a single fungal species from mixed growth. After initial isolation on Potato Dextrose Agar (PDA) plates, the plates were incubated at 25 ± 2°C for 5-7 days to allow for the development of fungal colonies. Once growth was established, distinct fungal colonies were visually identi ed based on their morphological characteristics such as color, texture, and growth pattern. A sterile cork borer was used to aseptically cut and transfer a circular disc from the actively growing margin of the selected fungal colony to a fresh PDA plate. This process helps in isolating the actively growing and uncontaminated portion of the fungus. The sub-cultured plates were incubated again under the same conditions until a uniform fungal growth was observed. The procedure was repeated many times to ensure complete puri cation and removal of any possible contaminants. The isolated fungal cultures were preserved at 4°C for subsequent identi cation and experimental applications.

4.2. Microscopy:

Microscopy is crucial in fungal identi cation as it allows for the detailed observation of morphological characteristics such as hyphal structure, spore formation, and reproductive bodies, which are key for distinguishing fungal species. Techniques like Lactophenol Cotton Blue, Calco uor White, and India Ink staining enhance visualization of fungal cell walls, spores, and capsules, aiding in differentiation from other microorganisms. The microscopic examination of the puri ed fungal isolates was performed using the Scotch tape preparation method with Lactophenol Cotton Blue (LPCB) staining. A clean glass slide was prepared by placing a single drop of LPCB stain in the center. A small piece of sterile transparent scotch tape was gently pressed onto the surface of a 7 day-old fungal culture to lift hyphae and spores.The adhesive tape bearing fungal structures was gently pressed, adhesive side down, onto a drop of Lactophenol Cotton Blue (LPCB) on a microscope slide to create a wet mount.

The slide was then examined under a compound light microscope at 100x magni cation.. The morphological features such as hyphal structure, conidiophores, and conidia were recorded to assist for identify the fungal isolates based on microscopic characteristics.

Molecular identi cation of puri ed fungal isolates was performed through ampli cation and sequencing of the Internal Transcribed Spacer (ITS) region of fungal rDNA, a highly conserved region used for fungal taxonomy and identi cation.

4.3.1 Fungal biomass preparation

A 5 mm disc from a puri ed fungal colony was cut using a sterile cork borer and inoculated into 100 mL of sterile Potato Dextrose Broth (PDB) in a 250 mL conical ask with Tween 80 to prevent clumping and promote uniform dispersion of fungal hyphae in the liquid medium. The culture was incubated at 25 ± 2°C with shaking at 120 rpm for 3 days, followed by static incubation for 4 days. After 7 days, the mycelial mat was crushed manually using a sterile mortar and pestle for DNA extraction.

4.3.2 Genomic DNA extraction

Genomic DNA was extracted utilizing the HiMedia® Fungal Genomic DNA Miniprep Puri cation Kit, following the protocol provided by the manufacturer. Initially, the fungal mycelium was grinded thoroughly using a rough mortar and pestle. Subsequently, a lysis buffer was applied to break down the cells. This step was complemented by enzymatic treatment to further facilitate cell disruption.DNA puri cation was carried out using a column-based protocol.. Finally, the puri ed DNA was eluted using an elution buffer and stored at -20°C for future use.The concentration and purity of the extracted DNA were assessed using spectrophotometric absorbance measurements.

4.3.3 PCR Ampli cation of ITS region

The ITS region of the rDNA was ampli ed using the ITS1 and ITS4 primers:

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')

ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

The PCR mixture was made by mixing the DNA template, forward and reverse primers, PCR master mix, and sterile distilled water in proper amounts.

25µL Hi-PCR® REDy Master Mix

1µL forward primer (ITS1).

1µL reverse primer (ITS4).

2.5µL of genomic DNA template.

Remaining volume made up with molecular-grade water to a nal volume of 50 µL.

The PCR was conducted using the following thermal cycling conditions:

Initial denaturation: 94°C for 5 minutes.

35 cycles of:

Denaturation: 94°C for 30 seconds

Annealing: 55°C for 30 seconds

Extension: 72°C for 1 minute.

Final extension: 72°C for 10 minutes

In 1.5%agarose gel with ethidium bromide, the samples were run to check the PCR result, in UV light, DNA bands had been checked.

4.4. Construction of Phylogenetic tree:

Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA v.11) software based on the Neighbour Joining method.

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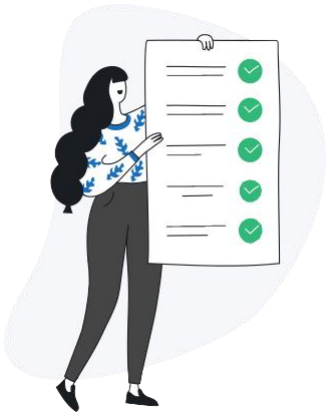

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Puri cation:
The puri cation process resulted in clear and distinct fungal colonies, as shown in the images. This con rms the effectiveness of the method in isolating pure fungal cultures from the mixed samples. Puri ed fungi are crucial for accurate identi cation and further study.
Microscopy:
By microscopic observation we have found the different types of IP in the septic or uncepted branch or cluster forms of the fungal isolates. These observations helped con rm the morphological identity of the fungi and supported the puri cation result
The above tables present DNA concentration data for infected (IRF) and healthy (HLF) tea leaf samples before and after gel puri cation. A increase in post-gel electrophoresis concentrations is observed in HLF samples, suggesting ef cient DNA recovery. The A260/A280 ratios (~1.6–1.9) and A260/230 ratios (~1.9–2.2) across both sets indicate good DNA purity, suitable for molecular applications like PCR and sequencing.
The evolutionary relationships among the fungal isolates were analyzed using ITS sequence data. The tree reveals distinct clustering of genera such as Trichoderma, Penicillium, Talaromyces, Fusarium, and Nectria, indicating genetic divergence among the isolates from healthy and infected tea samples. Branch lengths represent genetic distances, and closely related species are grouped together, re ecting their phylogenetic proximity.

Conclusion-
This study effectively revealed the complex fungal diversity associated with healthy and infected tea plants (Camellia sinensis L.), focusing on both leaves and roots, by employing a dual approach involving morphological and molecular techniques. Morphological examination was initially carried out using lactophenol cotton blue staining and the cellotape mount method, which provided foundational insights into the structural characteristics of the isolated fungi. However, since morphological identi cation can be limited by similarities in physical features among different fungal taxa, molecular characterization using Internal Transcribed Spacer (ITS) region ampli cation was adopted for accurate species-level identi cation.

The ITS-based sequencing and phylogenetic analysis unveiled a broad spectrum of fungal genera, re ecting the ecological diversity and functional roles of fungi in tea ecosystems. The identi ed fungi included Gelasinospora sp., Fusarium sp., Talaromyces sp., Penicillium sp., Nodulisporium sp., Trichoderma sp., and Nectria sp. Among these, several species are recognized pathogens, such as Fusarium solani and Fusarium proliferatum, which are known to cause dieback, collar canker, and leaf spot diseases. Additionally, Phomopsis theae and Poria hypolateritia were associated with collar stem canker and red root rot, respectively. These diseases are signi cant threats to tea plantations, leading

to reduced plant vigor, lower yield, and compromised leaf quality, which in turn affect both domestic markets and export potential.

Interestingly, the presence of multiple Trichoderma species, known for their antagonistic and biocontrol properties, highlights the coexistence of both pathogenic and bene cial fungi within the tea plant microbiome. These bene cial fungi may play a role in natural disease suppression, enhancing plant resilience and promoting healthier growth. Their identi cation provides potential for further exploration into their application as biological control agents against destructive fungal pathogens.

This research emphasizes the importance of integrating traditional fungal observation techniques with advanced molecular tools to achieve comprehensive mycological pro ling. Traditional methods remain valuable for preliminary screening, but molecular analysis allows researchers to overcome the limitations of culture-based identi cation, especially for non-sporulating, cryptic, or unculturable fungi.

Understanding the tea plant's mycobiome—the community of associated fungi—can lead to the development of more targeted and eco-friendly disease management strategies. By identifying not only harmful fungi but also bene cial and neutral endophytes, this study sets the stage for future research into plant-fungal interactions, microbial ecology, and their implications in sustainable agriculture.

Moreover, the insights gained from this study can assist in developing early detection systems for fungal pathogens, improving integrated pest management (IPM) protocols, and guiding breeders in selecting disease-resistant tea cultivars. These advances are especially crucial as climate change and intensive farming practices increase the vulnerability of crops to disease outbreaks.

In conclusion, this investigation provides a signi cant contribution to the understanding of fungal biodiversity in tea plants. It demonstrates how modern molecular biology tools, when combined with classical taxonomy, can unveil hidden microbial dynamics, ultimately aiding in enhancing tea crop health, productivity, and sustainability.

Plagiarized Sources

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